REMARKS

Claim 1 and 32-46 were pending in the application. Independent claims 1 and 39 have been amended to more fully and distinctly claim the present invention. Accordingly, claims 1 and 32-46 are currently pending in the present application. Support for the amendment to claims 1 and 39 to specify that the level of BCL-X_L protein is increased in the T cell is found, for example, at least at page 3, line 25-26 of the specification. Support for the amendment to claims 1 and 39 to recite a "mature" T cell is found in the specification, for example, at least on page 6, lines 29-31, and also by example, in Examples 1-5, which describe experiments that utilize mature T cells. Support for the amendment to claims 1 and 39 to recite "a submitogenic amount of an anti-CD3 antibody", is found, for example, at least in Example 1, which provides support by example in that submitogenic amounts of anti-CD3 antibody were contacted with T cells in the described experiments (discussed further below), and also in Example 8, at page 43, line 14-15 of the specification, which describes experiments in which submitogenic amounts of anti-CD3 antibody were contacted with T cells. Given the support for these amendments present in the specification as filed, no new matter has been added.

Attached hereto is Appendix A, captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**" The attached Appendix A contains a marked-up version of the changes made to the claims by the current amendment.

Amendment to the claims is not to be construed as acquiescence to any of the rejections set forth in the instant Office Action, and was done solely to expedite prosecution of the instant application. Applicants reserve the right to pursue the claims as originally filed, or similar claims, in this or one or more patent applications.

Claim Rejections Under 35 U.S.C. §112, first paragraph

Claims 1 and 32-46 have been rejected under 35 U.S.C.§112, first paragraph as not reasonably providing enablement for the instant claims. More specifically, the Examiner states:

> in light of numerous conflicting teachings in patents cited foregoing, the invention does not appear to be enabled absence of clarification of the contradictory evidence found in the references.

Applicants traverse and submit that the teachings in the specification and the cited patents are not conflicting, and present the following clarification for the benefit of the Examiner.

The Examiner appears concerned in the seemingly contradictory role of anti-CD3 antibodies in inducing or preventing programmed cell death of T cells, as reported in the art. The Examiner relies upon the disclosures of Nakai et al (US Patent No. 5,691,341) Kwon et al. (US Patent No. 6,303,121) Lenardo (US Patent No 6,083,503), and Example 6 of the instant application, as teaching that contacting anti-CD3 antibodies to a T cell induces programmed cell death. In response, Applicants submit that the seemingly contradictory effects of anti-CD3 antibodies on T cells cited by the Examiner is actually related to the type of T cell which is contacted. Applicants' claims specifically recite that the T cell is *ex vivo*, meaning from the body. Further, claims 1 and 39 have been amended to specify that the T cell is a "mature" T cell.

The disclosure of Nakai et al. refers to the phenomena known as activation-induced T cell death (AICD), known in the art to occur in ***immature T cells*** and also in some ***transformed*** cell lines and ***T cell hybridomas.*** The teaching of Nakai et al. that anti-CD3 induces programmed cell death in T cells is specific to ***immature T cells and hybridomas***. AICD does not result from contact of a mature, resting T cell obtained from an individual with anti-CD3 antibodies. Evidence of this is found in the articles cited by Nakai et al. in support of the statements (quoted by the Examiner) that apoptosis is inducible by anti-CD3 antibodies. Specifically, Smith et al. (*Nature 337*: 181-184 (1989), submitted herewith) and Tadakuma et al. (*Eur. J. Immunol. 20*: 279 (1990), submitted herewith), both teach that anti-CD3 antibodies induce ***mature*** T cells to proliferate, but induce ***immature*** thymocytes to die via apoptosis. Similarly, the disclosure of Kwon et al. also refers to AICD as occurring in immature T cells, hybridomas and transformed cell lines. Further to this distinction, Example 6 of the instant application describes experiments performed on Jurkat cells, which are of a ***transformed cell line*** and subject to AICD. In contrast, Examples 1-5 utilize primary T cells obtained from donor individuals, which are not subject to AICD.

The claims, as amended, which recite that the T cell is ***ex vivo***, and is a ***mature T cell***, clearly exclude immature T cells, transformed T cells and hybridomas, which are reported to undergo AICD upon exposure to anti-CD3 antibodies. Applicants therefore submit that the claims as amended may be performed by of one of ordinary skill in the art with no more than routine experimentation.

The Examiner further cites the disclosure of Kwon et al. as teaching that "continued presence of anti-Cd3 in cell culture would cause T cell unresponsiveness to even saturated anti-CD28 [levels]" resulting in cell death. Applicants submit that reactivation multiple times taught by Kwon et al. is not equivalent to continued presence of anti-CD3 as recited by the Examiner. Applicants submit that the disclosure of Kwon et al. teaches that activated T cells which are ***reactivated*** multiple times with ***high doses*** of anti-CD3 can become resistant to the effects of CD28, and anti-CD28. However, in an effort to expedite prosecution, Applicants have amended the instant claims to recite "***sub-mitogenic*** amounts of anti-CD3". The teachings of Kwon et al., with respect to anti-CD3 inducing apoptosis, are specific to ***high doses*** of anti-CD3 and thus use of the submitogenic amounts of anti-CD3 antibodies to protect a T cell from programmed cell death is clearly are enabled.

The Examiner further suggests that the claims are not enabled with respect to cells previously exposed to IL-2, in light of the teachings of Lenardo that IL-2 causes T cells to undergo apoptosis upon re-immunization with an antigen. Applicants submit that the teachings of Lenardo do not indicate that the instant claims will not work. Lenardo teaches a specific effect of IL-2 exposure of T cells *in vitro* prior to antigen exposure, wherein subsequent re-exposure of the T cells to antigen within 2-3 days *in vitro* leads to T cell death. Lenardo further teaches that "the effects of IL-2 wear off 2-3 days after IL-2 is no longer present, hence rechallenge must occur within that period"(column 12, line 27-29). Since the effects of IL-2 exposure wear off relatively quickly, given the guidance of the specification and the knowledge of one skilled in the art, one so skilled could practice the claimed methods without inadvertently inducing cell death in cells which may have been previously exposed to IL-2.

With respect to superantigens, the Examiner argues that some superantigens have been observed to cause apoptosis, rather than protect T cells from apoptosis, as in the claimed method, citing Johnson et al., Lenardo, and Lynch et al. Applicants submit that the cited teachings of Johnson et al. are highly speculative, and are not supported by experimental evidence. With respect to the teachings of Lenardo, that bacterial Staphylococcus superantigen induced T cell PCD in mice, and the teaching of Lynch et al. that certain antigens and superantigens cause T cell deletion in LPR mice, Applicants point out that the reported phenomena occur *in vivo*. No evidence is presented that apoptosis would occur as a result of contact of the superantigens under controlled conditions *ex vivo*. The claimed methods are performed under controlled conditions,

to produce the desired inhibition of cell death of the T cell isolated from an individual. Applicants have presented evidence that a variety of superantigens can protect a mature T cell from cell death when utilized in the claimed methods, and have enabled a representative number of species for the claimed genus. Moreover, the determination of additional superantigens appropriate for use in the claimed methods is within the ability of one of ordinary skill in the art through no more than routine experimentation.

The Examiner further argues that polyclonal activators such as lectins are not enabled by the specification for use in the claimed methods, citing the teachings of Kwon et al. as teaching use of " PHA as a trigger to induce PCD in T cells." In response, Applicants argue that Kwon et al. teaches activation of T cells with the lectin phytohemagluttinin, followed by induction of apoptosis of the activated T cells by repeated activation by anti-CD3 and anti-CD28 (column 18, lines 18-20). These teachings do not indicate that Applicants' claims are not enabled. The Examiner also cites the disclosure of Schlossman et al. as teaching "treatment with anti-CD3 antibody, ionomycin, and/or phorbol ester can induce apoptosis in both human and mouse immature thymocytes" and further "culturing PBT cells with PMA-treated monocytes in medium containing ionoycin or PMA increased the level of apoptosis by almost 3- and 6- fold, respectively." Applicants submit that Schlossman et al. teach a monocyte dependent activation of apoptosis in T cells, wherein the monocyte (or antigen presenting cells) are primed by exposure to phorbol myristate acetate or phytohaemagglutinin. Schlossman et al. further teach that phytohaemagglutinin may be used to activate antigen presenting cells to induce their ability to prime resting T cells for apoptosis (column 4, line 24-32 and claim 5). Again, these teachings do not indicate that Applicants' claims are not enabled. It is within the ability of one of ordinary skill in the art to practice the claimed invention given the guidance presented in the application and the knowledge of one skilled in the art.

Regarding claims 39-46, the Examiner indicates that the claims will be evaluated by the standard of *in vivo* application, and states:

> the art is still unpredictable with regard to particular cell types and agents that enhance Bcl-X_L levels. ...many other cell surface receptors, apoptotic associated molecules, environmental factors also play a role in the state of the T cells, the specification fails to teach with regard to *in vivo* aspects of the invention, the influence of other factors, the disease and the disorder that would need T cell protection, whether and how long the protective effect of in vitro T cell treatment would last. In view of such, the fact of

treated T cells is highly unpredictable in a complicated in vivo environment, thus resulting in a trial and error situation.

The Examiner's concern is largely based on the fact that the teachings of the specification are obtained in cell culture, and on the alleged unpredictability of the art with regard to cell types and agents that enhance BCL-X_L levels, citing for support the disclosure of Boise et al. (Cell 75:597-608 (1993)) Gottschalk et al., (Proc. Natl. Acad. Sci. USA 91: 7350-7354 (1994)) and Roberts (US Patent No. 5,686,281). Applicants submit that the art is not as unpredictable as the Examiner suggests and that the Examiner has failed to provide teachings in the art that indicate a lack of enablement of Applicants' specific claimed invention. For instance, the disclosure of Boise et al. is cited by the Examiner as teaching that "six hours of stimulation with PMA and ionomycin had no effect on bcl-x mRNA expression in double-positive thymocyte populations but induced a dramatic increase in bcl-x mRNA expression in both single-positive thymocytes and peripheral blood T cells." Applicants argue that these teachings ***support*** enablement of the present claims, as amended, which specify ***mature*** T cells, whereas double positive thymocytes (immature T cells), taught by Boise et al. to be unaffected by PMA and ionomycin with respect to bcl-x expression, are excluded. Thus, the findings of Boise et al. are consistent with the claimed methods.

The Examiner further cites the disclosure of Gottschalk et al. as teaching that "cyclosporin A, FK-506, and rapamycin could prevent PCD in T-cell hybridomas and thymocytes, but induce PCD in B cells," in support of the argument regarding the influence of other factors on the treated T cell. With respect to the teachings of Gottschalk et al., Applicants point out that these teachings are not contradictive to the effectiveness of the claimed methods since the claims are directed to methods for protecting ***T cells*** from apoptosis. The teachings of Gottschalk et al., as per the comments of the Examiner that "many other cell surface receptors, apoptotic associated molecules, environmental factors also play a role in the state of the T cells," have no bearing on the instant claims.

The Examiner further cites the disclosure of Roberts as teaching the involvement of other cell surface receptors, apoptotic associated molecules, and environmental factors that play a role in the state of the T cells. In response, Applicants argue that the teachings of Roberts in no way indicate lack of enablement of the claimed methods. The disclosure of Roberts teaches chimeric

receptors used to genetically engineer immune cells to respond to costimulation through the chimeric receptors, e.g., to prevent the induction of anergy in the recipient cells. These teachings have no bearing on the instant claims.

Regarding the above quoted comments of the Examiner that the specification fails to teach the disease and the disorder that would need T cell protection, Applicants submit that the ordinary skilled artisan will understand that the claimed methods are useful in treatment of an individual in need of an increased immune response, e.g., individuals infected with pathogens which cause T cell death, or for boosting an immune reaction in order to more rapidly eliminate an infection (page 32, line 7-8). Applicants direct the Examiner's attention to page 30, line 37-39 of the specification, which indicates that the methods of the present invention are useful for preventing cell death of CD4+ T cells of an HIV infected individual and protecting T cells from HIV infection. Further to this, Example 9 of the application details experiments that indicate bcl-X_L expression can prevent HIV-1-induced cell death.

Regarding the concerns of the Examiner of the duration of the protection conferred to the T cell upon reintroduction into the subject, Applicants again argue that the Examiner has failed to provide teachings in the art that indicate that the duration conferred would be insufficient for useful treatment, and further argue that even a short lived effect of apoptosis resistance of the T cell so administered could provide beneficial results to an individual, and that if necessary said treatment could be repeated to produce optimal effects. Moreover, under certain circumstances, a short lived duration would be preferred (see for example, page 32, line 18-21).

In summary, Applicants have amended the claims to clearly indicate that the invention is drawn to the use of "mature T cells", and also to recite that "submitogenic amounts of anti-CD3 antibody" are used. With respect to the amended claims, the application clearly teaches the use of the claimed methods, such use being credible to those of ordinary skill in the art. Applicants therefore respectfully request reconsideration and withdrawal of the rejection.

Claim Rejection Under 35 U.S.C. §102

Claim 1 has been rejected under 35 U.S.C. 102(e) as being anticipated by US Patent 6,352,694. Applicants note that this patent is referred to by the Examiner as "Thompson et al.," however in fact the inventors listed on this patent are actually June et al. This response is

therefore made with the assumption that the Examiner meant to cite June et al., US Patent No. 6,352,694. The Examiner states that June et al.:

> "teach a method comprising contacting T cells *in vitro* with an anti-CD3 antibody (step a of claims 1 and 17 of the cited patent), then an anti-CD28 antibody (step b of claim 1 of the cited patent) or a CD28 ligand selected from B7-1 or B7-2 (step b of claim 17)" and also that "T cells could be used in treating infectious disease and cancer, which embrace HIV (claims 15, 16, 31, and 32)."

In response, Applicants point out that protection from apoptosis is not necessarily coincident with induction of cell proliferation, and likewise, a culture which is protected from apoptosis, is not necessarily proliferating. Indeed, a proliferating cell culture may still be undergoing a significant amount of apoptosis. Applicants have identified a function of anti-CD3 in T cells which is independent of its proliferative activity. In Example 1 of the instant application, Applicants show that use of a submitogenic amount of an anti-CD3 antibody is sufficient for protection from programmed cell death, but not sufficient to induce proliferation upon costimulation. Applicants refer the Examiner to Example 1, page 34, line 25- which reads:

> The maintenance of cell viability in anti-CD3 stimulated and anti-CD3 + anti-CD28 stimulated cells was not the result of subsequent T cell proliferation as cell counts done in parallel to the viability assays revealed that the absolute cell number did not change. Furthermore, all the cells in the activated populations were arrested within the cell cycle at either late G1 or G2.

The protective activity of anti-CD3 is therefore separable from the proliferative activity of anti-CD3. Moreover, claims 1 and 39 have been amended to specify a ***submitogenic*** of amount of anti-CD3 antibody which is not taught or suggested by June et al. Applicants therefore request withdrawal of the rejection.

Claim Rejection Under 35 U.S.C. §103

Claims 1, 34, and 38 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Lenardo in view of Roberts. More specifically, the Examiner states:

> It would have been obvious for one skilled in the art to combine the teachings of Lenardo and Roberts in the prevention of T cell from undergoing apoptosis in cultures with a reasonable expectation of success. The ordinary skilled artisan would have been motivated to do so because the addition of two protecting agents would enhance the protective effects for T cells, and IL-2 is a commonly used cytokine in T cell cultures.

This rejection is respectfully traversed on the grounds that the references, either alone or in combination, do not result in Applicants' claimed invention. Lenardo is cited as teaching that "blockage of IL-2 receptor (contacting IL-2 or agonist) reverses bacterial superantigen induced T cell PCD". Roberts is cited as teaching anti-CD28 antibodies protect T cells from apoptosis *in vitro*. In contrast to the Examiner's assertions, Lenardo does not teach contacting IL-2 to a T cell to prevent apoptosis. Lenardo teaches ***blockade*** of IL-2, ***not activation*** of the IL-2 receptor. Contacting the IL-2 receptor with IL-2 leads to activation of the IL-2 receptor. One of ordinary skill in the art would not reasonably interpret the term "blockade," as it is used in the teachings of Lenardo, to encompass contacting the IL-2 receptor with IL-2. Roberts does not supply the missing elements from the Lenardo patent. The claims are therefore not taught or suggested by either reference, alone or in combination.

Double Patenting

Claims 1, 34, 37, and 38 have been rejected under the judicially created doctrine of obvious-type double patenting as being unpatentable over claims 1, 15-19, 31, and 32 of U.S. Patent No. 6,352,694 (June et al.). More specifically the Examiner states:

> Although the conflicting claims are not identical, they are not patentably distinct from each other because claims 1, 34, 37, and 38 of the present application and claims 1, 15-19, 31, and 32 of the cited patent are each drawn to a method comprising the steps of contacting the T cell with agents, i.e. anti-CD3 antibody, and anti-CD28 antibody, or a CD28 ligand, wherein the T cells are HIV infected (infectious and cancerous).
>
> The processes of the present application and the cited patent differ from the other in the preamble recitations, however, the recitations "for inducing a population of T cell" in the cited patent or "for protecting a T cell from cell death" in the present application are obvious variants, i.e. T cells in a healthy growing state are resistant to apoptosis.

In response to the above quoted comments by the Examiner, Applicants reiterate the above stated argument that a proliferative population of cells is not necessarily resistant to apoptosis. Moreover, in an effort to expedite prosecution, claims 1 and 39 have been amended to specifically recite a ***submitogenic amount*** of anti-CD3. Applicants thus submit that the currently pending claims are patentable over June et al. and request withdrawal of this rejection.

CONCLUSION

In light of the above amendments and remarks, entry of the above amendment and reconsideration of the application is requested. If a telephone conversation with Applicants' attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 227-7400.

Respectfully submitted,

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Dated: **February 4, 2003**

APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Changes made to the Amended Claims:

1. (Amended) A method for protecting a mature T cell from cell death, comprising contacting the T cell *ex vivo* with at least two agents selected from the group consisting of an anti-CD28 antibody, a sub-mitogenic amount of an anti-CD3 antibody, an anti-CD2 antibody, a CD28 ligand, interleukin-2 (IL-2), ionomycin, A23187, phorbol-12, 13-dibutyrate, a lectin and a superantigen, wherein the agent increases [which augments] BCL-X_L protein level in the T cell such that the T cell is protected from cell death.

39. (Amended) A method for protecting a mature T cell from cell death in a subject by increasing [augmenting] the level of BCL-X_L protein in said T cell, comprising obtaining said T cell from said subject, contacting said T cell *ex vivo* with at least two agents selected from the group consisting of an anti-CD28 antibody, a sub-mitogenic amount of an anti-CD3 antibody, an anti-CD2 antibody, a CD28 ligand, interleukin-2 (IL-2), ionomycin, A23187, phorbol-12, 13-dibutyrate, a lectin and a superantigen, and reintroducing said T cell into said subject, such that T cell death is inhibited in said T cell of said subject.

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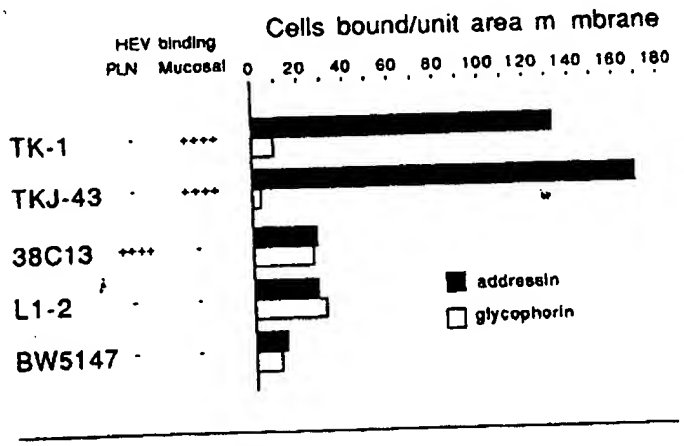

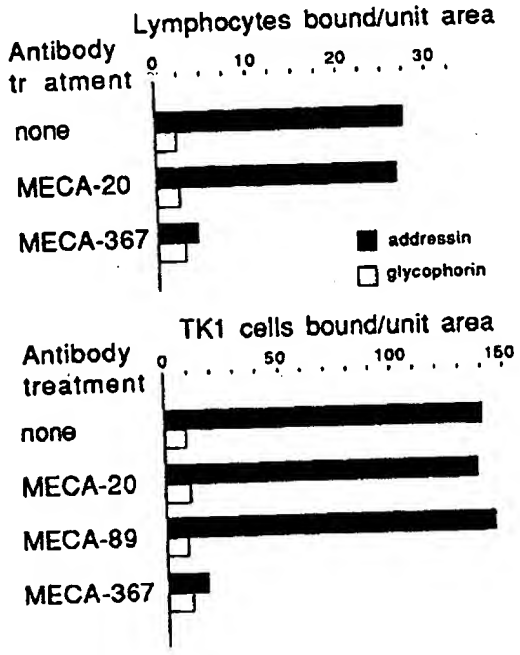

Fig. 4 Lymphocyte binding to MAD is inhibited by antibody MECA-367 but not by control antibodies.
Methods. Binding of lymphocytes or TK1 cells to the membranes was carried out as in Fig. 2, except that the membranes were preincubated with 50 $\mu g\ ml^{-1}$ MECA-367, MECA-89 or class-matched control antibody MECA-20 (ref. 17) (against an irrelevant endothelial cell antigen) in RPMI, 10 mM HEPES for 15 min, and then rinsed before application of sample cells. The bars represent pooled data from independent experiments with similar results, except for the binding of TK1 cells to untreated or MECA-89-treated glycophorin-reconstituted membranes, which was assayed only once. Comparable background-level binding was observed on untreated or MECA-89-treated control membranes lacking inserted protein (not shown). One of the two experiments examining TK1 binding to untreated membranes is shared with Fig. 3.

determining cell positioning or cell targeting in other systems, f r example in the development of specific n uronal connections during neurogenesis, or the migration of differentiating embryonic cell populations during morphogenesis.

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Fig. 3 Binding of lymphomas to the mucosal addressin correlates with their ability to bind to mucosal HEV.
Methods. The lymphomas used here have been described[6,12,13]. Briefly, TK-1 and TKJ-43 are AKR/Cum and AKR/J thymic lymphomas respectively; these lymphomas were passaged *in vivo*, frozen in liquid nitrogen from recipient lymph nodes, and used immediately after thawing. 38C13, a C3H/eb B-lineage lymphoma[16], was obtained directly from the lymph nodes of adoptive recipients. L1-2, a C57L pre B cell line, and BW5147, an AKRT cell lymphoma, were grown *in vitro*. The HEV-binding characteristics of these lymphomas have been described[6,12,13] and are summarized in the figure. Because the HEV-binding of cell lines can vary, however, these binding properties were confirmed by assaying the particular lymphoma preparations used here in the *in vitro* frozen-section assay[2,6,12]. As reported, TK1 and TKJ43 bound substantially better than reference normal lymphocytes to Peyer's patch HEV, 38C13 bound better than normal lymphocytes to peripheral lymph node HEV, and L1-2 and BW5147 were non-binders. Lymphoma binding to MAd- or glycophorin-containing membranes was as in Fig. 2. The data represent the mean results of 1-3 experiments performed on different days.

Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures

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The receptors found on most T lymphocytes bind to antigen presented on major histocompatibility complex proteins and consist of dimers of α- and β-polypeptides associated with the invariant CD3 complex[1,2]. A fully competent immune system requires a diverse array f T-cell antigen receptors (TCRs) with different specificities. This diversity is generated by rearrangement of TCR α- and β-chain gene segments within the thymus[3,4] where the receptors are first expressed. Any cells carrying self-reactive receptors must be eliminated, suppressed or inactivated so that destructive autoimmunity is avoided. Recently, compelling evidence has shown that ne process involved in producing such self-tolerance is clonal deleti n of autoreactive cells within the thymus by an

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as-yet-undefined mechanism[5–8]. Here we show that engaging the CD3/TCR complex of immature mouse thymocytes with anti-CD3 antibodies produces DNA degradati n and cell death through the endogen us pathway of apoptosis. Activati n of this process in immature T cells by the binding of the TCR to self-antigens may therefore be the mechanism which produces cl nal deleti n and consequently self-tolerance.

Anti-CD3 antibodies induce mature T cells to proliferate[9], but immature thymocytes, expressing low levels of surface CD3, do not proliferate in response to these antibodies[10,11]. But anti-CD3 antibodies do elicit a transitory increase in cytoplasmic calcium concentration in immature thymocytes, although the magnitude of the response is smaller than that of mature cells[11]. As thymocytes can activate an endogenous suicide mechanism which can be triggered directly by a number of stimuli, including changes in intracellular calcium concentration[12,13], we investigated whether engaging the CD3/TCR complex with anti-CD3 antibodies could result in the activation of this mechanism. We studied this response in organ cultures of fetal mouse thymus, which provide an *in vitro* system that can support TCR gene rearrangement and expression[4] and avoid the problem of low cell viability encountered in cultures of isolated thymocytes. The TCR-positive cells make up ~50% of the lymphocytes in such cultures (ref. 14, and our unpublished results) and are at an immature stage of development (see legend to Fig. 1).

Antibodies to mouse CD3 added for the last 18 hours of culture resulted in a substantial reduction in cell yield (~45%, see Table 1) and induced the degradation of DNA to oligonucleosomal bands which is characteristic of apoptosis[12,13] (Fig. 1, lanes 3 and 4). Toluidine-blue stained sections (Fig. 2*a*) and cytospins of anti-CD3 treated organ cultures showed numerous apoptotic thymocytes. The chromatin condensation and cell shrinkage characteristic of apoptotic cells was also clearly shown by electron microscopy (Fig. 2*b*). The cell death and DNA degradation response of immature thymocytes was produced by both hamster anti-CD3 and rat anti-CD3 antibodies. The same response was also observed in chicken embryo thymus organ cultures treated with anti-chicken CD3 antibodies (unpublished work with M. D. Cooper). In contrast, the same rat and hamster anti-CD3 antibody supernatants strongly stimulated proliferation when incubated with mature T cells from mouse spleen (data not shown), as has been previously reported[9]. The response of T lymphocytes to incubation with anti-CD3 antibodies was therefore critically dependent on the stage of maturity of the cells: the same antibody treatment which caused splenic T cells to proliferate caused immature T cells to die. Significantly, depletion of the double positive $CD4^+CD8^+$ cells in organ cultures treated with anti-CD3 antibody accounted for most (83%) of the reduction in cell number (Table 1).

The effects of antibodies reacting with several other T-cell surface markers were analysed to investigate the possibility that apoptosis was only induced by antibodies reacting with the CD3/TCR complex. A rat anti-Thy 1 antibody also produced stimulation of mature T cells (data not shown), but did not induce significant DNA degradation in immature thymocytes (Fig. 1, lane 2). Rat anti-CD4 antibody of the same subclass (IgG2b) as the rat anti-CD3 antibody also failed to induce apoptosis (Fig. 1, lane 5) despite the observation from fluorescence-activated cell sorting that the immature thymocytes bound substantially m re anti-CD4 antibody than anti-CD3 antibody (data not shown). Thy-1 and CD4 are displayed by a large majority of such immature $CD3^+$ cells[10].

W tested the hypothesis that cells displaying surface receptors were eliminated by the anti-CD3 antibody, whereas cells displaying only cytoplasmic TCR β-chains were unaffected. We used monoclonal antibody F23.1 (ref. 15), that reacts with the products f the Vβ8 gene family and therefore with some 20% of BALB/c TCRs[15] and can be used to detect both surface and cytoplasmic Vβ8-chain expression[16]. Immunostaining of anti-CD3 antibody treated thymus organ cultures revealed that F23.1 surface-positive cells had indeed been almost completely removed (0.9%, compared with 5.0% in controls), but that the proportion of cells containing only Vβ8-chains in the cytoplasm remained at 9.5 to 10.5%. It has previously been shown that incubation of thymic organ cultures with such antibodies to particular Vβ gene products specifically eliminates cells bearing corresp nding surface receptors[16].

The effects of plasma-membrane receptor stimulation of a number of cell types can be reproduced by treatment with phorbol myristate acetate (PMA) and the calcium i nophore, ionomycin[17,18]. These components seem to act by mimicking the increase in cytoplasmic calcium c ncentration and protein kinase C activity which result from stimulation f cell-surface

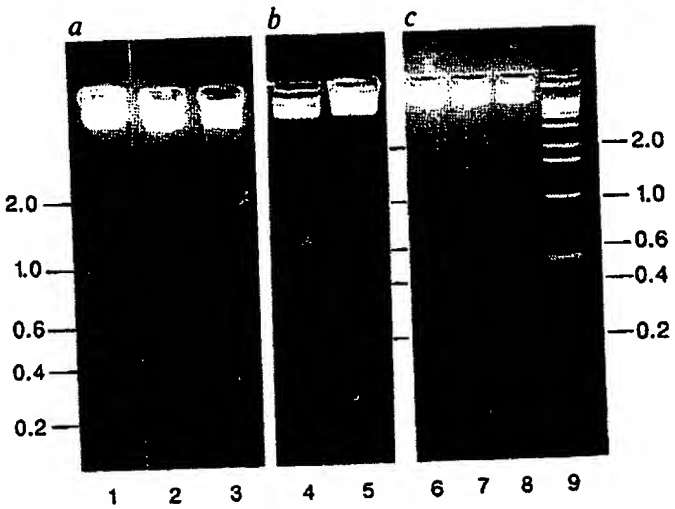

Fig. 1 Gel analysis of thymocyte DNA from cultured thymic lobes. Total DNA from 10^6 cells after treatment of cultures with (1) anti-mouse I-A^b (negative control; mouse IgG2a hybridoma 25-9-175[25]), (2) anti-mouse Thy-1 (rat IgG2a hybridoma G7[24]), (3) anti-mouse CD3 (hamster hybridoma 1452C11[9]), (4) anti-mouse CD3 (rat IgG2b hybridoma 29B; Portole *et al.*, personal communication), (5) anti-mouse CD4 (rat IgG2 hybridoma YTS 191.1[23]), (6) ionomycin (800 ng ml^{-1}) and PMA (10 ng ml^{-1}), (7) ionomycin (800 ng.ml^{-1}), (8) PMA (10 ng ml^{-1}), (9) molecular size standards (BRL). Molecular sizes in kilobases are indicated on the left for the gel in *a* and on the right for gels in *b* and *c*. Oligonucleosomal fragments appear as ladders of diffuse bands whose molecular sizes are approximately multiples of 200 base pairs.

Methods. BALB/c (I-A^d) fetal thymus lobes isolated at day 14 of gestation were allowed to develop in organ culture on the surface of nucleopore filters supported on gelatin foam sponges, as described previously[16]. After 7 days of culture, surface TCR expression can be detected[16] and 91% of all lymphocytes present (96% of TCR-positive cells) also express J11d, which is found on immature but not on mature T cells[22]. On day 7 of culture, the medium was replaced with a 50:50 mixture of fresh medium and the required hybridoma supernatant, or with fresh medium containing the indicated concentrations of ionomycin and/or PMA. After further incubation overnight (~18 h), the lobes were collected, washed and teased apart in ice-cold medium with cataract knives to liberate the lymphoid component. Portions of $\sim 10^6$ cells were washed and pelleted as previously described[26], except that the centrifugation was carried out at 4 °C. Pellets were resuspended in 20 µl 10 mM EDTA, 50 mM Tris-HCl (*p*H 8.0) containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg ml^{-1} proteinase K, and incubated at 50 °C for one hour. 10 µl 0.5 mg ml^{-1} RNase A was added to each sample and incubation at 50 °C continued for a further hour. Samples were heated to 70 °C, and 10 µl 10 mM EDTA (*p*H 8.0) containing 1% (w/v) low-gelling-temperature agarose, 0.25% (w/v) Bromophenol blue and 40% (w/v) sucrose was mixed with each sample before loading into the dry wells of a 2% (w/v) agarose gel containing 0.1 µg ml^{-1} ethidium bromide using a siliconized pipette tip. Electrophoresis was carried out in 2 mM EDTA 800 mM Tris-phosphate (*p*H 7.8) until the marker dye had migrated 3–4 cm.

Table 1 Phenotypes of cells in antibody-treated thymus organ cultures

Antibody present	$CD4^+CD8^+$ Yield	%	$CD4^+CD8^-$ Yield	%	$CD4^-CD8^+$ Yield	%	$CD4^-CD8^-$ Yield	%
None	100,800	44.6	17,600	7.8	14,900	6.6	92,600	41.0
Anti-I-A^b	88,700	45.5	13,400	6.9	10,900	5.6	81.900	42.0
Anti-CD3	16,300	15.1	9,800	9.1	8,500	7.9	73,000	67.9

Yields are given in cells per lobe and percentages of total cells in the lobe. The overall yield of cells was reduced by 45% in anti-CD3 antibody-treated lobes relative to anti-I-A^b antibody-treated lobes (negative control) which were incubated at the same time. Fetal thymus lobes were incubated for six days before the addition of either anti-I-A^b antibody[25] or hamster anti-CD3 antibody[9] and incubation for a further 18 h (as described in Fig. 1). Lobes were then teased to release cell suspensions (20 lobes per treatment) which were double-labelled for CD4 and CD8 expression by sequential incubation with monoclonal rat anti-CD4 (ref. 23), followed by goat anti-rat immunoglobulin-fluorescein. They were then washed with 1% normal rat serum to block any free anti-rat combining sites and incubated with monoclonal rat anti-CD8-biotin (Becton-Dickinson) with 1% rat serum, followed by avidin-D Rhodamine (Vector Labs). Absence of cross-reactivity was demonstrated by the presence of single positive $CD4^+$ or $CD8^+$ cells only in purified lymph node T cells. In all cases at least 300 cells were scored using an epi-illumination Zeiss microscope.

receptors. This combination strongly stimulates the proliferation of mature T cells[10] and, as anti-CD3 antibodies induce an increase in cytoplasmic calcium in immature T cells[10,11], we tested the effect of PMA and ionomycin on immature thymocytes in organ culture. Figure 1*c* shows that ionomycin together with PMA strongly induces apoptosis in immature thymocytes, producing the characteristic DNA degradation pattern (lane 6). PMA alone had no detectable effect (lane 8), but treatment with ionomycin alone (lane 7) was apparently no less effective than when it was combined with PMA, suggesting that the increase in intracellular calcium concentration alone was sufficient to induce the cell suicide response. The DNA degradation observed in response to ionomycin alone was similar to that reported for A23187, a calcium ionophore with somewhat different specificity, on rat thymocytes incubated *in vitro*[12].

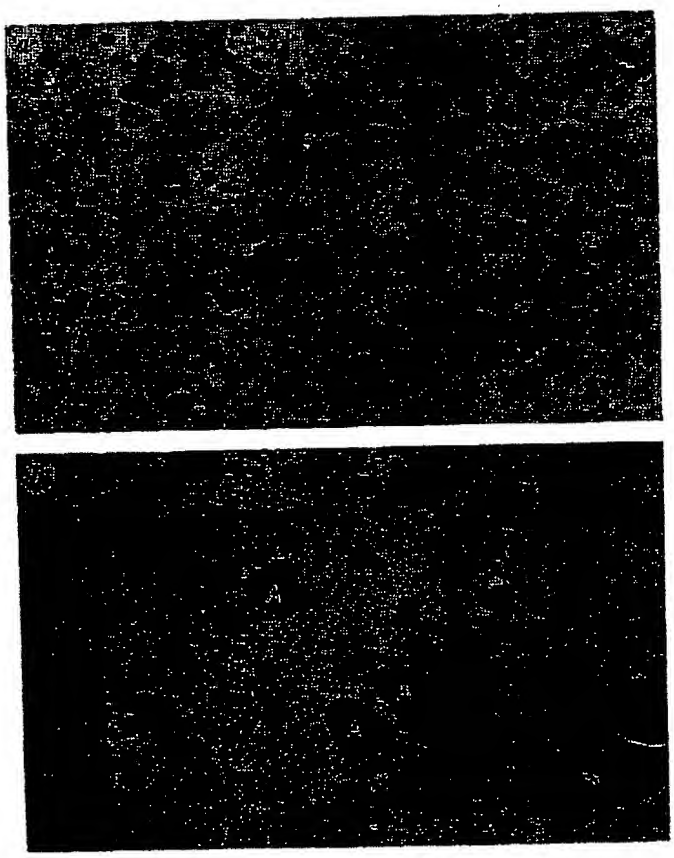

Fig. 2 Histological appearance of cells induced to undergo apoptosis in intact fetal thymus lobes after short-term exposure to anti-CD3 (as described in Fig. 1). *a*, Toluidine blue-stained 1 μm sections. *b*, Electron micrograph taken from the same anti-CD3 treated culture as in *a*. *A* and *N* indicate representative apoptotic and normal lymphocytes respectively. Note the highly condensed

In the studies of Kappler *et al.*[5-7] and MacDonald *et al.*[8], which demonstrated clonal deletion of autoreactive cells expressing receptors with affinity for I-E or Mls, it was noted that immature cortical cells with receptors of the appropriate specificity were present, but that mature medullary cells were absent. These results indicated that the deletion process normally occurs between the immature and mature phases, although a recent report showed that $CD4^+CD8^+$ immature TCR-positive cells can also be deleted in some circumstances[19]. Here we have shown that immature $CD4^+CD8^+$ cells are sensitive to the induction of apoptosis by anti-CD3 antibodies. The reason for this apparent discrepancy could be that autoreactive immature cortical cells in the thymus *in vivo*, although sensitive to deletion, are not normally deleted until they contact antigen on dendritic antigen-presenting cells, which are located throughout the medulla and at the cortico-medullary junction but not in the cortex. Indeed, other studies have indicated that tolerance induction within the thymus depends on contact with dendritic cells[20,21].

Whatever the natural interactions in the intact thymus which lead to clonal deletion, our results clearly show that engagement of the TCR complex of immature thymocytes can result in the induction of apoptosis. In view of the potency of the effect of anti-CD3 antibodies (as compared with anti-CD4 antibodies f the same isotype), it seems unlikely that the effect is mediated by opsonization or antibody-dependent cell killing involving other cells. Rather, we suggest that the effect is a direct one on developing T cells which respond to a signal (which would stimulate mature T cells) by degrading their DNA and undergoing cell suicide. Final proof of the relevance of this mechanism in clonal deletion will depend on the direct demonstration of apoptosis in developing T cells which are in contact with the antigen(s) for which they have affinity.

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An Fc receptor structurally related to MHC class I antigens

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Maternal immunoglobulin G transmitted to the fetus or newborn provides humoral immunity for the first weeks of mammalian life. Fc receptors on intestinal epithelial cells of the neonatal rat (FcRn) mediate the uptake of IgG from milk. Affinity-purified FcRn is resolved by SDS-PAGE into components of relative molecular masses 45,000–53,000 (p51) and about 14,000 (p14)[1–3]. We report the identification of the smaller component as β_2-microglobulin. Association of β_2-microglobulin with p51 was confirmed by cross-linking in intestinal epithelial cell brush borders. We have cloned a cDNA encoding the presumptive Fc-binding subunit, p51, and its predicted primary structure has three extracellular domains

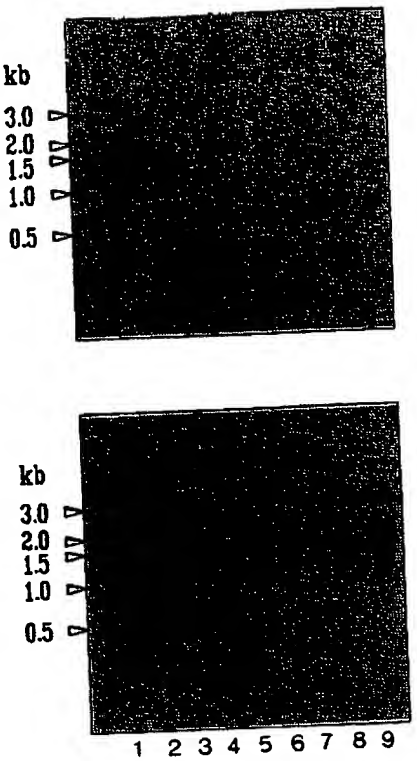

Fig. 2 Northern blot of RNA from neonatal and adult rat tissues, probed with a 925-bp cDNA from a clone immunoselected with antiserum to the FcRn large subunit, p51. Nucleotide identities of 50–55% between p51 and some MHC class I sequences may explain the detection of hybridizing mRNAs in tissues that do not transport IgG. Lane 1, neonatal proximal small intestine; 2, neonatal distal small intestine; 3, adult small intestine; 4, adult brain; 5, adult heart; 6, adult kidney; 7, adult liver; 8, adult pancreas; 9, adult spleen. **Methods.** RNAs (25 μg total RNA, except adult small intestine: 1 μg poly[A]$^+$RNA) were fractionated by electrophoresis in 1.5% agarose/formaldehyde gels and transferred to nylon membranes (Gene Screen Plus, NEN). Hybridization was in 5× SSC, 50% formamide at 42 °C with a [^{32}P]DNA probe primed with random hexanucleotides from the 925-bp cDNA (cloning methods as in Fig. 3 legend). The membrane was washed in 0.1× SSC at 50 °C, air-dried and exposed at −70 °C to pre-flashed Kodak XAR 5 with an intensifying screen for 8 h (top) and 36 h (bottom).

Fig. 1 *a*, Radioiodinated FcRn immunoprecipitated with antisera to p51, p14 and β_2m, analysed by SDS-PAGE. *b*, Immunoblot analysis of FcRn prepared after crosslinking of brush border proteins Relative molecular masses are shown in thousands. **Methods.** *a*, We isolated brush borders from epithelial cells of the proximal third of the small intestine of 11-day-old Wistar rats (Charles River). Membrane proteins were solubilized in 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS) in 50 mM sodium phosphate, *p*H 6.5, and cycled over an affinity matrix of human or rat IgG coupled to agarose. The physiological *p*H-dependence of IgG binding[27] allowed us to elute $Fc_\gamma R$ by shifting the *p*H from 6.5 to 8.0 (ref. 2). Affinity-purified FcRn was labelled with ^{125}I-labelled Bolton & Hunter reagent. We raised antisera in New Zealand White rabbits against the putative receptor subunits, using gel slices containing p51 or p14 cut out after SDS-PAGE as immunogen. Aliquots of [^{125}I]-FcRn were incubated with rabbit antisera to p51, p14 or human β_2m or with normal rabbit serum (nrs), overnight at 4 °C in PBS/1 mgml^{-1} CHAPS/1 mgml^{-1} ovalbumin, *p*H 7.4. At this *p*H IgG does not bind its receptor through Fc. Protein A-Sepharose was added to precipitate immune complexes. The beads were washed in PBS/1 mgml^{-1} CHAPS. Bound proteins were eluted by boiling in gel sample buffer containing 5% SDS and 5% 2-mercaptoethanol, then electrophoresed on 7–14% acrylamide gradient gels. The gels were dried and exposed at −70 °C to pre-flashed Kodak XAR 5 for 24–48 h with intensifying screens. *b*, Brush borders were incubated in suspension with the thiol-cleavable crosslinking reagent dithiobis(succinimidyl propionate) (DSP, 1 mM from a 100× stock in dimethylformamide) for 4 h at 4 °C. 50 mM ethanolamine, *p*H 8.0, was added to quench the reaction. Control samples were prepared by adding dimethylformamide alone. The brush borders were collected by centrifugation. Membrane proteins were extracted and affinity chromatography was done as before. Crosslinked and control samples were analysed by electrophoresis under reducing and non-reducing conditions. We electroblotted the separated proteins onto nitrocellulose and probed the filters with anti-p51 or anti-human β_2m. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a second layer and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazole.

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CD4+CD8+ thymocyt s are susceptible to DNA fragmentation induced by phorbol ester, calcium ionophore and anti-CD3 antibody

Stimulation of murine thymocytes with phorbol ester or calcium ionophore for 18–24 h resulted in 70%–80% fragmentation of DNA into 180–200-bp multiples, followed by cell death. Experiments with fractionated subpopulations by panning or flow cytometry revealed that DNA fragmentation was selectively observed in $CD4^+CD8^+$ cells and in a portion of $CD4^-CD8^+$ cells. To investigate whether DNA cleavage is also inducible via antigen-specific receptors, thymocytes were incubated in wells precoated with anti-CD3 antibody. An approximately 20% increase of DNA fragmentation was constantly observed when unseparated thymocytes were stimulated with anti-CD3 antibody. In this anti-CD3-induced DNA degradation, $CD4^+CD8^+$ cells are probably the target cells, since (a) fetal thymocytes at day 18 of gestation were found vulnerable to anti-CD3-induced DNA cleavage and (b) flow cytometry analysis of viable cells recovered after cultivation in the anti-CD3-coated wells revealed that $CD4^+CD8^+$ cells were preferentially decreased. Further experiments with purified $CD4^+CD8^+$ cells, however, could not define a clear-cut increase of DNA fragmentation when isolated $CD4^+CD8^+$ cells were stimulated with anti-CD3 antibody. Addition of interleukin (IL) 1, IL 2, IL 3, IL 4 or interferon-γ to the $CD4^+CD8^+$ cell cultures failed to yield a DNA cleavage similar to that of unseparated thymocytes.

1 Introduction

During the maturation and differentiation in the thymus, autoreactive thymocytes are suspected to be deleted especially at the stage of $CD4^+CD8^+$ cells [1–6], but the elimination mechanism has not been clarified. We recently found that the murine thymocytes stimulated with PMA (5 ng/ml) or with calcium ionophore A23187 (200 nM) resulted in 70%–80% of DNA fragmentation into 180–200-bp multiples as compared to 15%–20% fragmentation in control cultures without stimulation. In contrast, the concurrent addition of PMA and A23187 greatly reduced DNA fragmentation and a massive proliferation was observed. The onset of DNA degradation requires a lag period of several hours, and this fragmentation was completely blocked by actinomycin D or cycloheximide (CHX), indicating that newly synthesized mRNA and one or more proteins are necessary for this phenomenon. The increase of DNA fragmentation induced by PMA or A23187 was not observed in peripheral T cells obtained upon nylon wool purification of LN cells. Furthermore, when thymocytes were separated by peanut aggulutinin (PNA), DNA fragmentation was pronounced in PNA^{high} thymocytes, but marginal in PNA^{low} thymocytes. These results suggest that immature thymocytes are susceptible to DNA fragmentation [7].

In this report, we show that DNA fragmentation induced by PMA or A23187 is preferentially observed in $CD4^+CD8^+$ cells. We further describe that anti-CD3 antibody can induce DNA cleavage and that the $CD4^+CD8^+$ cells are the target cells of DNA fragmentation. However, when the isolated $CD4^+CD8^+$ cells are stimulated with anti-CD3 antibody, an increase of DNA fragmentation is not clearly defined. Several interpretations of this last point are given and our results are discussed with reference to negative selection mechanism operating in the thymus.

2 Materials and methods

2.1 Reagents

PMA, 4'6-diamidino-2-phenylindole and CHX were purchased from Sigma (St. Louis, MO). Calcium ionophore A23187 was obtained from Calbiochem (La Jolla, CA), PMA and A23187 were stocked in DMSO and diluted appropriately in ethanol when used.

2.2 Mice

C57BL/6 mice were obtained from our animal facilities and were used at the age of 5 to 8 weeks for preparation of thymocytes. Fetal thymocytes from day 18 of gestation were also obtained from C57BL/6 mice.

2.3 Antibodies

Anti-CD4 mAb (rat anti-mouse L3T4, GK1.5; [8]) and CD8 (rat anti-mouse Ly-2, 53-6.72; [9]) were gifts from Dr. H. Nariuchi (Tokyo University). Mouse anti-Ly-2.2 (83-12-5), rat anti-mouse Ly-2.2 (2.43; [10]) and anti-CD3 (145-2C11; [11]) mAb were kindly supplied from Drs. D. H. Sachs (NCI, NIH, Bethesda), Y. Katsura (Kyoto University) and M. Minami (Tokyo University), respectively. Mouse

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Abbreviations: CHX: Cycloheximide

anti-rat serum was obtained by immunizing C57BL/6 mice with rat IgG. The mAb were purified on a protein A-Sepharose column (Pharmacia, Uppsala, Sweden). FITC-labeled anti-CD8 and PE-labeled anti-CD4 antibodies were purchased from Becton Dickinson (Tokyo, Japan).

2.4 Preparation of thymocyte subpopulations

Thymocytes were separated into $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ cells by panning (Fig. 1a) or sorting by FCM (Fig. 1b). To prepare thymocyte subpopulations by panning, the following methods were used. For preparation of $CD4^+CD8^+$ cells, thymocytes (5×10^7 cells/ml) were treated with rat anti-Ly-2 mAb (53-6.72) at a 1:50 final dilution ascites fluid. Thereafter $CD8^+$ cells were collected by panning after a 1-h incubation at 37 °C on 60-mm dishes (# 25010; Corning Glass Works, Corning, NY) or 100-mm dishes (# 3003; Falcon, Oxnard, CA) which were pre-coated with 1 mg/ml of mouse anti-rat IgG. $CD8^+$ cells were then transferred to anti-L3T4 antibody (GK1.5; 1 mg/ml)-coated dishes, and $CD4^+CD8^+$ cells were collected as adherent cells after a 1-h incubation. In the experiment of Figs. 3, 4 and 5, preparation of $CD4^+CD8^+$ cells was modified and the cells were prepared by sequentially adhering onto anti-Ly-2.2 (2.43)-coated dishes and anti-L3T4 (GK1.5)-coated dishes at 4 °C. $CD4^+CD8^-$ and $CD4^-CD8^-$ cells were prepared from thymocytes treated twice with anti-Ly-2.2 (83-12-5) at a final dilution of 1:100 of ascites fluid plus 1:20 rabbit complement (Pel-Freez, WI), followed by panning on GK1.5-coated dishes. $CD4^-CD8^+$ cells were prepared from nonadherent cells absorbed twice on GK1.5-coated dishes. The cells were then treated with anti-Ly-2 mAb and recovered as adherent cells from dishes which were pre-coated with mouse anti-rat antibody. The purity of subpopulations was checked indirectly by FCM analysis after staining with FITC-labeled anti-rat IgG, PE-labeled anti-CD4 or FITC-labeled anti-CD8, and it was estimated to be about 95%. Alternatively, thymocyte subpopulations were prepared by cell sorting on FACStar (Becton Dickinson). $CD4^+CD8^+$ cells were sorted directly from thymocytes stained with FITC-labeled anti-CD8 and PE-labeled anti-CD4. $CD4^+CD8^-$ and $CD4^-CD8^-$ cells were obtained from thymocytes, which had been pre-treated twice with anti-Ly-2.2 plus C, after staining with PE-labeled anti-CD4. $CD4^-CD8^+$ cells were sorted from cells which did not adhere to GK1.5-coated dishes and which were further stained with FITC-labeled anti-CD8.

2.5 Activation of thymocytes with PMA, A23187 and anti-CD3 antibody

Unseparated and separated thymocytes were suspended in RPMI 1640 complete medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), adjusted to 4×10^6 cells/ml and distributed into 35-mm dishes (Falcon 3001) in a volume of 2 ml. PMA and A23187 were appropriately diluted in ethanol, and added to the cultures at 5 ng/ml (PMA) or 200 nM (A23187) in volumes of 10 µl. Control cultures always received 10 µl of ethanol. For the stimulation with anti-CD3 and anti-CD4 mAb, 24-well plat s (# 143982; Nunc, Roskilde, Denmark) were coated with 0.5 ml of protein A-purified antibodies (25 µg/ml or 100 µg/ml) at 37 °C for 60 min, followed by PBS containing 1% FCS at 37 °C for 60 min. Unfractionated or fractionated thymocytes were incubated in these wells in 1 ml for 18 h.

2.6 DNA cleavage assay

Eighteen hours later, the cells were harvested from one dish or two wells as one sample and DNA cleavage was examined by the procedure described previously [12] with the additional modifications of Wyllie et al. [13]. Briefly, the cell pellets were lysed in 5 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.5% Triton X-100 for over 20 min on ice. All the thymocytes were lysed under these conditions. The lysate and its SN (after centrifugation at 27000 × g for 20 min) were sonicated for 1 min and then DNA contents were measured by a fluorometric method using 4',6-diamidino-2-phenylindole. The percentage of DNA fragmentation was defined as the ratio of DNA content in the SN to that of the whole lysate, and the data represents the mean of two samples or the mean ± SD of three samples.

2.7 Agarose gel electrophoresis of DNA

Unseparated thymocytes, $CD4^+CD8^+$ cells, $CD4^-CD8^-$ cells and $CD4^+CD8^-$ cells were cultured with or without 5 ng/ml of PMA for 18 h. The cells were harvested, lysed with SDS and treated with proteinase E (Kaken Drug Co., Tokyo, Japan) for 4 h at 37 °C in the presence of RNase A (Sigma; 100 µg/ml) and RNase T_1 (Sigma; 240 U/ml). DNA was extracted with phenol/chloroform, precipitated in 70% ethanol and then dissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The same amount of DNA samples (4 µg) was applied to 1.8% agarose gels and gels were stained with ethidium bromide. Molecular size markers are mixtures of Hind III digest of λ-phage DNA and Hae III digest of ΦX174 (Nippon gene; Nippon, Tokyo, Japan).

3 Results

3.1 DNA fragmentation induced by PMA and A23187 is preferentially observed in $CD4^+CD8^+$ cell

By using anti-CD4 and anti-CD8 mAb thymocytes were separated into four subpopulations. As shown in Fig. 1a, the increased of DNA fragmentation was remarkable in $CD4^+CD8^+$ cells, but not in $CD4^-CD8^-$ cells or $CD4^+CD8^-$ cells, although background levels in $CD4^-CD8^-$ cells were higher than in other populations. In contrast to $CD4^+CD8^-$ cells, it is noteworthy that a significant increase of DNA fragmentation was constantly observed in $CD4^-CD8^+$ cells. The differences in the extent of DNA fragmentation were not due to the direct effects of mAb, since (a) addition of anti-CD4 and/or anti-CD8 mAb alone did not induce a DNA fragmentation greater than background level in total thymocytes (data not shown), (b) positively selected $CD4^+CD8^-$ cells as well as a mixed population of $CD4^+CD8^-$ and $CD4^-CD8^-$ cells, negatively selected by anti-Ly-2.2 plus C and therefore having no

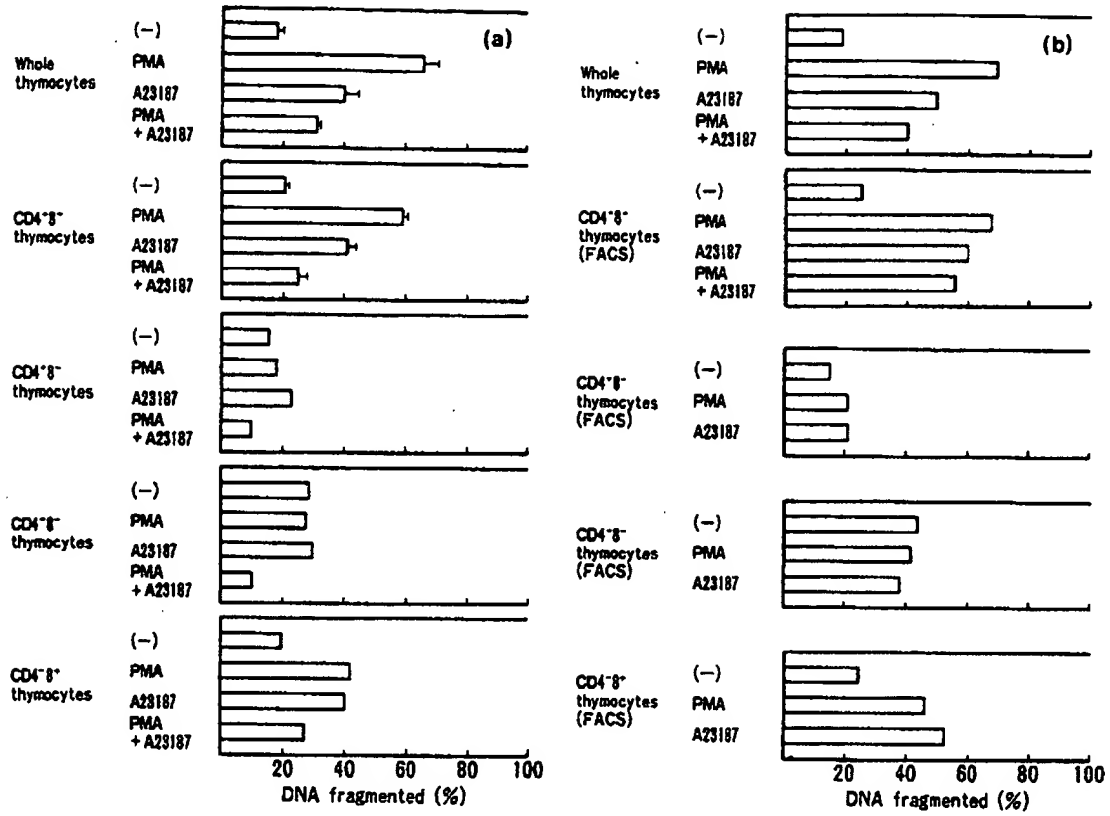

Figure 1. DNA fragmentation induced by PMA or calcium ionophore A23187 in the subsets of thymocytes. Thymocytes were fractionated into $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ cells by panning (a) or FCM (b) as described in Sect. 2.4. The cells were stimulated with 5 ng of PMA and/or 200 nM of A23187. Eighteen hours later, the cells were harvested and DNA fragmentation was assessed. The data represents the mean of two samples or the mean ± SD of three samples.

expected antibody attached to them, did not show an increased fragmentation upon stimulation and (c) the extent of DNA fragmentation was not significantly altered when total thymocytes were stimulated with PMA or A23187, in the presence or absence of anti-CD4 and/or anti-CD8 mAb.

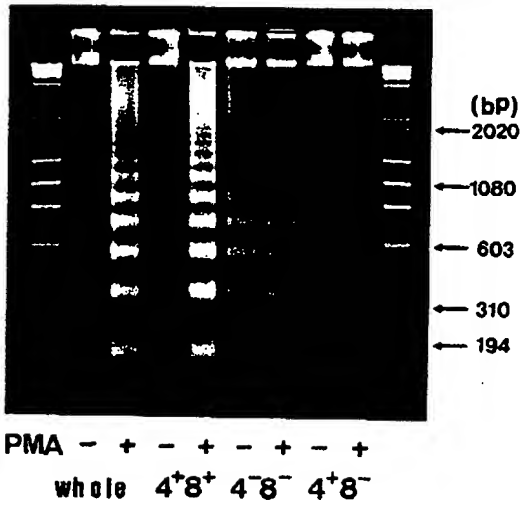

Figure 2. Agarose gel electrophoresis of DNA extracted from thymocyte subpopulations after stimulation with PMA. Unseparated thymocytes, $CD4^+CD8^+$ cells, $CD4^-CD8^-$ cells and $CD4^+CD8^-$ cells were cultured with (+) or without (−) 5 ng/ml of PMA for 18 h. DNA was extracted from each group and 4 μg was applied to 1.8% agarose gel.

These results were confirmed by using thymocyte subpopulations fractionated by FCM (Fig. 1b). The background levels were rather high, probably due to the longer period needed for cell sorting, but the results obtained were quite similar. Again, a slight but significant increase in DNA fragmentation was observed in $CD4^-CD8^+$ cells.

To confirm that DNA was actually fragmented into 180–200-bp multiples, unfractionated thymocytes, $CD4^+CD8^+$ cells, $CD4^-CD8^-$ cells and $CD4^+CD8^-$ cells were incubated with or without PMA for 18 h, and DNA was extracted from each subpopulation. Equal amounts of DNA (4 μg) were electrophoresed in 1.8% agarose gel. Fig. 2 clearly demonstrates that fragmentation of DNA was accelerated in unfractionated thymocytes and $CD4^+CD8^+$ cells when stimulated with PMA. In contrast, the extent of fragmentation was not much different between stimulated and unstimulated cells in $CD4^-CD8^-$ or $CD4^+CD8^-$ subpopulations, although the background levels were slightly different in each population.

3.2 Anti-CD3 antibody can induce DNA fragmentati n in unseparated thymocytes

About half of $CD4^+CD8^+$ cells express lower levels of TcR/CD3 complex [14] than do $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. It is interesting to investigate whether DNA fragmentation is inducible by signals via the TcR/CD3 complex, since elimination m chanisms of self-reactive thymocytes should be acting via antigen receptors. When unfraction-

ated thymocytes were incubated in wells pre-coated with anti-CD3 antibody (25 or 100 μg/ml), an approximately 20% increase of DNA fragmentation was constantly obtained (Fig. 3). As observed in PMA- or A23187-induced DNA degradation, the anti-CD3-induced fragmentation was blocked in the presence of CHX (25 μg/ml), suggesting that a similar activation step is operative in both cases. In contrast, the cells incubated in anti-CD4 antibody (25 or 100 μg/ml)-coated wells did not yield an increase of DNA fragmentation, indicating that physical binding of thymocytes to antibody-coated dishes is not the sole cause of DNA fragmentation. FCM analysis of thymocytes after a 24-h cultivation in the presence of anti-CD3 revealed that approximately 35% of the cells were dead (as assessed by propidium iodide) as compared to around 20% dead cells in control cultures without stimulation. The number of viable $CD4^+CD8^+$ cells recovered after cultivation was constantly less (20%–30%) in anti-CD3-stimulated cultures than in control cultures (data not shown). Furthermore, fetal thymocytes at day 18 of gestation, in which $CD4^+CD8^+$ cells were dominant, were found vulnerable to DNA cleavage (see Fig. 5, Sect. 3.3). These results strongly suggest that $CD4^+CD8^+$ cells are the main target cells susceptible to DNA fragmentation induced by anti-CD3 antibody.

3.3 Attempts to induce DNA fragmentation in the isolated $CD4^+CD8^+$ cells with anti-CD3 antibody

We next tried to confirm the above notion by utilizing $CD4^+CD8^+$ cells fractionated by a panning procedure. However, the increase of DNA fragmentation was not as clearly defined as shown in Fig. 3. We have tried to elucidate

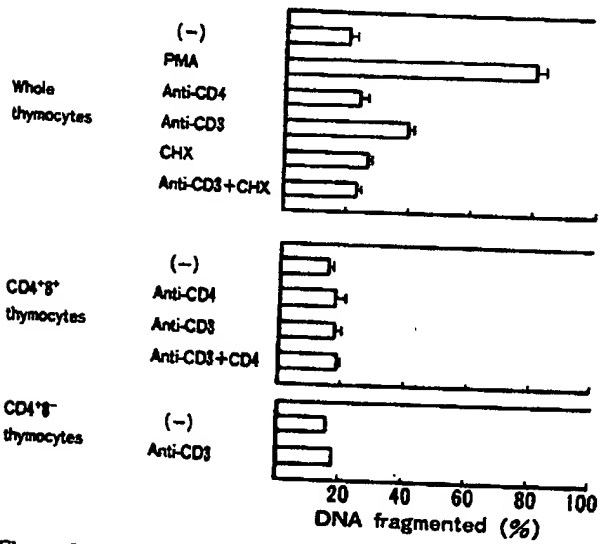

Figure 3. DNA fragmentation induced by anti-CD3 mAb. Twenty-four-well plates were coated with 0.5 ml of protein A-purified anti-CD3 antibody or anti-CD4 antibody (100 μg/ml) at 37 °C for 60 min, followed by PBS containing 1% FCS at 37 °C for 60 min. Unfractionated thymocytes, $CD4^+CD8^+$ cells or $CD4^+CD8^-$ cells at 4×10^6 cells/ml were incubated in these wells in 1 ml for 18 h. PMA (5 ng/ml) and CHX (25 μl/ml) were added at the start of culture. The cells collected from two wells were pooled as one sample, lysed and the DNA fragmentation was assessed. The data are not shown here, but similar results were obtained in the experiments with antibodies coated at 25 μg/ml.

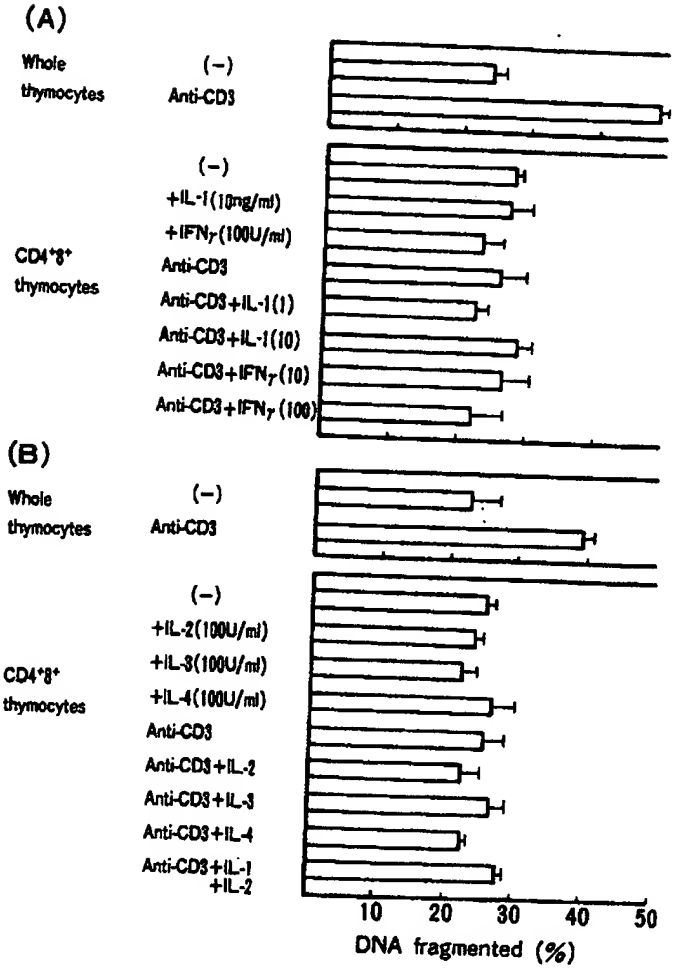

Figure 4. Stimulation of isolated $CD4^+CD8^+$ cells with anti-CD3 antibody in the presence of various cytokines. (A) The isolated $CD4^+CD8^+$ cells were incubated in the anti-CD3-coated wells together with IL 1 (1, 10 ng/ml), and IFN-γ (10, 100 U/ml). After 18 h, the cells were harvested and the extent of DNA fragmentation was assessed. (B) $CD4^+CD8^+$ cells were incubated with anti-CD3 antibody in the presence of IL 2 (100 U/ml), IL 3 (100 U/ml) or IL 4 (100 U/ml), or a combination of IL 1 (10 ng/ml) and IL 2 (100 U/ml). The data were not included here, but similar results were obtained with 10 and 1000 U/ml of IL 2, 10 U/ml of IL 3, or 10 and 1000 U/ml of IL 4.

why the increase of DNA cleavage was ill-defined when purified $CD4^+CD8^+$ cells were used. First of all, it is possible that signals acting via the TcR/CD3 complex are not sufficient and that co-operation with some other cells or with factors released from anti-CD3-activated thymic cells are necessary for the induction of DNA fragmentation in $CD4^+CD8^+$ cells. To address this possibility, several factors including IL 1 (1, 10 ng/ml), IL 2 (10, 100, 1000 U/ml), IL 3 (10, 100 U/ml), IL 4 (10, 100, 1000 U/ml), and IFN-γ (10, 100 U/ml) were added to the culture of $CD4^+CD8^+$ cells, but a significant increase of DNA fragmentation was not obtained (Fig. 4). We have tested, however, only a limited number of cytokines, and the possible involvement of other cytokines or of cell-to-cell interactions remains to be investigated.

Alternatively, it is also possible that DNA cleavage is inducible solely by the signaling via the TcR/CD3 complex but that the binding of anti-CD3 antibody is inadequate for the delivery of activation signals leading to DNA degrada-

tion. Recently, it has been suggested that an efficient signaling via the TcR/CD3 complex requires co-clustering with CD4 or CD8 molecules [15, 16]. CD4+CD8+ cells express low levels of TcR/CD3 complex and the positively selected cells inevitably retain antibodies to CD4 and CD8 molecules on the surface. These antibodies may interfere with co-clustering of TcR/CD3 and CD4/CD8 and, thus, weaken the TcR/CD3-mediated signaling. In the course of these studies, we noticed that the density of CD3 antigen on the surface of CD4+CD8+ cells rapidly increased merely by culturing them for a short period such as 4 h (see also [17]). We investigated whether signaling via TcR/CD3 alone is adequate for inducing DNA degradation in CD4+CD8+ cells when the signal is strengthened by the augmented surface expression of CD3. CD4+CD8+ cells were prepared from fresh thymocytes or from thymocytes pre-cultured for 4 h, and the extents of DNA degradation were compared after incubation in the wells coated with anti-CD3 antibody (Fig. 5). Apparently, CD4+CD8+ cells prepared from pre-cultured thymocytes were more susceptible to fragmentation. The extent of fragmentation, however, was still less than that of unseparated thymocytes. These results suggest that the level of surface expression will be one of the important factors for the CD3-mediated triggering of DNA cleavage in CD4+CD8+ cells.

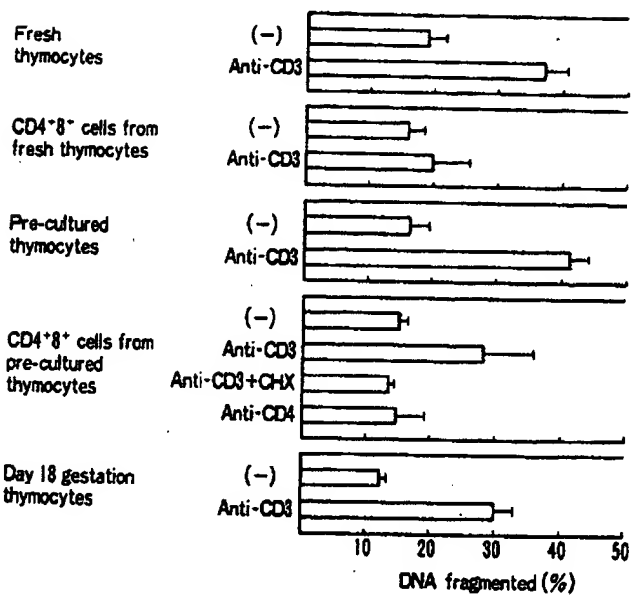

Figure 5. DNA fragmentation induced by anti-CD3 antibody in CD4+CD8+ cells which were prepared either from fresh thymocytes or from thymocytes pre-cultured at 37 °C for 4 h. CD4+CD8+ cells from two sources (a) from fresh thymocytes and kept at 4 °C in Hanks' solution containing 10% FCS after preparation, or (b) from thymocytes precultured at 37 °C for 4 h were adjusted to 4×10^6 cells/ml, and 1 ml of each cell suspension was incubated in the wells pre-coated with anti-CD3 mAb. CHX was added at 25 μg/ml. Furthermore, fetal thymocytes from day 18 of gestation were obtained and the DNA fragmentation induced by anti-CD3 antibody was investigated. In this particular experiment, fetal thymocytes included 17% of CD4−CD8−, 75% of CD4+CD8+, 2% or CD4+CD8− and 6% of CD4−CD8+ cells, and almost all of the CD3+ cells belonged to dully stained immature populations (data not shown).

4 Discussion

We demonstrate here that there is a critical stage susceptible to PMA- or A23187-induced DNA fragmentation during thymocyte differentiation, particularly at the stage of CD4+CD8+ cells. In addition, a slight but significant increase in DNA cleavage was constantly observed in CD4−CD8+ cells. With respect to this point, it is interesting to note that CD4−CD8+ but not CD4+CD8− cells have been described to be separatable into two mutually exclusive subpopulations by the expression of an immature thymocyte marker, J11d [18] and CD3. We have not tested, however, whether J11d+ immature CD4−CD8+ cells are responsible for the PMA- or A23187-induced DNA fragmentation.

The increase of DNA fragmentation was also inducible by the signaling via the TcR/CD3 complex, and the circumstantial evidence strongly suggested that CD4+CD8+ cells are the target cells. Further experiments with purified CD4+CD8+ cells, however, did not reveal a clear-cut increase in DNA fragmentation. There are several possibilities to explain this discrepancy. First, it is possible that the induction of DNA fragmentation requires the cooperation with some other cells or with cytokines produced by these cells. We tested various cytokines including IL 1, IL 2, IL 3, IL 4 and IFN-γ, but their addition to the CD4+CD8+ cell cultures failed to yield a DNA cleavage similar to that of unseparated thymocytes. Second, there is a possibility that antibodies bound to the CD4 or CD8 antigens during the preparation of CD4+CD8+ cells will physically block the binding of anti-CD3 antibody to the CD3 antigen. However, at least as judged by staining with FITC-labeled anti-CD3 antibody such blocking was not observed (data not shown). Third, an efficient signaling via the TcR/CD3 complex may require co-clustering with CD4 or CD8 molecules [15, 16], and antibodies to CD4 and CD8 antigens may interfere with co-clustering of TcR/CD3 and weaken the TcR/CD3-mediated signaling. Finally, antibodies bound to CD4 and CD8 antigens have also been described to interfere [19, 20] with TcR/CD3-mediated signaling, and it might be possible that the antibodies bound to positively selected CD4+CD8+ cells would have a negative influence on the anti-CD3-induced DNA fragmentation. At present, all of these possibilities are still open, and further experiments will be necessary to investigate the signals required for the induction of DNA fragmentation.

It is clear from our experiments that the signaling via the TcR/CD3 complex can induce DNA fragmentation of thymocytes *in vitro*. We could not, however, detect the fragmentation of DNA in thymocytes when those were prepared from thymus immediately after excision, and, thus, we have no direct evidence that this DNA fragmentation phenomenon, a characteristic feature of apoptosis [21], is physiologically relevant to the negative selection mechanism in the thymus. Several reports have indicated that the antigen receptors of autoreactive T cells appear to be expressed at one point at the stage of CD4+CD8+ cells [1–5, 22–24], and that they are then eliminated upon contact with dendritic cells located at the cortico-medullary junction [24, 25]. Therefore, the incidence of apoptosis *in vivo* would be rather low and hard to detect. Along these lines, DNA fragmentation *in vivo* might be easier to detect in a model system utilizing transgenic mice in which massive numbers of self-reactive T cells are eliminated.

Recently, Smith et al. [26] reported that treatment with anti-CD3 antibody induced DNA fragmentation in immature thymocytes. Their experimental system consists of organ culture and gel electrophoresis, and therefore is not adequate to see the extent of DNA fragmentation or the effects on the subpopulation of thymocytes. Nonetheless, their experiments also support that $CD4^+CD8^+$ cells are susceptible to DNA cl avage induced by anti-CD3 antibody. They further described that calcium ionophore but not PMA could induce DNA fragmentation, claiming that the increase in intracellular calcium concentration alone was sufficient to induce the cell suicide response. In contrast to these results, we clearly demonstrated here that PMA alone could induce DNA cleavage and that the combination of PMA and calcium ionophore greatly diminished the fragmentation. It is so far not clear whether the discrepancy between our results and theirs is due to the difference in the experimental systems. In addition, Shi et al. [27] reported that administration of anti-CD3 antibody in mice could induce DNA fragmentation of thymocytes even *in vivo*. Their results further support that apoptosis might be one of the mechanisms for negative selection. We should point out, however, that experiments with anti-CD3 antibody still contain some artifacts, and to attain conclusive evidence it will be necessary to demonstrate that DNA fragmentation is inducible via the antigen receptors in contact with specific antigens and MHC products.

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